A1 Hubert Köster; Dirk Van den Boom, filed July 10, 2000, entitled "METHODS FOR GENERATING DATABASES AND DATABASES FOR IDENTIFYING POLYMORPHIC GENETIC MARKERS".

Please replace the paragraph on page 5, lines 2-19, with the following paragraph:

Previously unidentified alleles of the human AKAP10 gene are provided. One allele, designated AKAP10-5 contains a previously undisclosed single nucleotide polymorphism (SNP), an A-to-G transition, at nucleotide position 2073 of the AKAP10 gene coding sequence. This SNP is located in the C-terminal PKA binding domain, and results in an Ile-to-Val substitution at the protein level for the AKAP10 gene protein product. Another allele, designated AKAP10-6 contains a previously undisclosed single nucleotide polymorphism (SNP), a C-to-G transversion, at nucleotide position 83587 of the human chromosome 17 sequence (SEQ ID NO: 17). The AKAP10 gene is located at approximately nucleotide position 83,580 to nucleotide position 156,577 of the chromosome 17 sequence. This SNP is located in the 5' untranslated region and 132 nucleotides upstream of the translation start site. A further allele, designated AKAP10-7 contains a previously undisclosed single nucleotide polymorphism (SNP), a G-to-A transition, at nucleotide position 129,600 of the human chromosome 17 sequence. This SNP is located four bases 3' to the exon 10/intron 10 boundary of AKAP10 mRNA.

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Please replace the paragraph on page 10, lines 14-16, with the following paragraph:

A3 Further provided are primers that specifically hybridize at a position immediately adjacent to a position corresponding to position 83587 of SEQ ID NO: 13 or 17 of an AKAP10 allele.

Please replace the paragraph on page 12, lines 13-15, with the following paragraph:

A4 Further provided are primers that specifically hybridize at a position immediately adjacent to a position corresponding to position 129600 of SEQ ID NO: 14 or 17 of an AKAP10 allele.

Please replace the paragraph on page 15, lines 5-8, with the following paragraph:

A5 Also provided are methods where the nucleotide detected at a position corresponding to position 2073 is a G and where the nucleotide detected at the complement of a position corresponding to position 2073 is a C.

Please replace the paragraph on page 17, lines 7-8, with the following paragraph:

A6 Also provided is a cell that contains a heterologous nucleic acid, that encodes the amino acid sequence set forth in SEQ. ID. NO: 4.

Please replace the paragraph beginning on page 29, line 17, through page 30, line 8, with the following paragraph:

A7 In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. (See, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to

skilled artisans (Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Human Genome Computing, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988). Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al., Nucleic Acids Research 12(I)*:387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J Molec Biol 215*:403 (1990)).

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Please replace the paragraph on page 32, lines 5-12, with the following paragraph:

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Thus, by "isolated" is meant that the nucleic acid is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) immediately flank the gene encoding the nucleic acid of interest. Isolated DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a native DNA sequence, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

Please replace the paragraph on page 34, lines 3-4, with the following paragraph:

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As used herein, the term "conjugated" refers stable attachment, such as ionic or covalent attachment.

Please replace the paragraph beginning on page 34, line 28, through page 35, line 3, with the following paragraph:

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As used herein, "indicating" or "determining" means that the presence or absence of an allelic variant may be one of many factors that are considered

when a subject's predisposition to a disease or disorder is evaluated. Thus a predisposition to a disease or disorder is not necessarily conclusively determined by only ascertaining the presence or absence of one or more allelic variants, but the presence of one of more of such variants is among a number of factors considered.

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Please replace the paragraph beginning on page 39, line 27, through page 40, line 13, with the following paragraph:

As used herein, "signal transduction" refers to the propagation of a signal. In general, an extracellular signal is transmitted through the cell membrane to become an intracellular signal. This signal can then stimulate a cellular response. The term also encompasses signals that are propagated entirely within a cell. The polypeptide molecules involved in signal transduction processes are typically receptor and non-receptor protein kinases, receptor and non-receptor protein phosphatases, nucleotide exchange factors and transcription factors. One of the key biochemical mechanisms involved in signal transduction is protein phosphorylation. AKAP10 proteins are involved in signal transduction as they bind to protein kinase A (PKA) and are thought to anchor the kinase at a location, *e.g.*, the mitochondria, where PKA acts to phosphorylate a specific substrate. Thus, an alteration in AKAP10 binding to PKA, localization to the mitochondria, or phosphorylation by PKA, among other steps will result in an alteration in signal transduction. Assays including those that determine phosphorylation by PKA, association of PKA and AKAP10 and localization of AKAP10 can be used to monitor the state of signal transduction.

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Please replace the paragraph beginning on page 49, line 18, through page 50, line 17, with the following paragraph:

d. Nucleic acid sequencing-based methods

In one embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence at least a portion of an AKAP gene and

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to detect allelic variants, *e.g.*, mutations, by comparing the sequence of the sample sequence with the corresponding wild-type (control) sequence. Exemplary sequencing reactions include those based on techniques developed by Maxam and Gilbert (Proc. Natl. Acad. Sci. USA (1977) 74:560) or Sanger (Sanger *et al.* (1977) Proc. Natl. Acad. Sci 74:5463). It is also contemplated that any of a variety of automated sequencing procedures may be used when performing the subject assays (Biotechniques (1995) 19:448), including sequencing by mass spectrometry (see, for example, U.S. Patent Nos. 5,547,835, 5,691,141, and International PCT Application No. PCT/US94/00193 (WO 94/16101), entitled "DNA Sequencing by Mass Spectrometry" by H. Köster; U.S. Patent Nos. 5,547,835, 5,622,824, 5,851,765, 5,872,003, 6,074,823, 6,140,053 and International PCT Application No. PCT/US94/02938 (WO 94/21822), entitled "DNA Sequencing by Mass Spectrometry Via Exonuclease Degradation" by H. Köster), and U.S. Pat. Nos. 5,605,798, 6,043,031, 6,197,498, and International Patent Application No. PCT/US96/03651 (WO 96/29431) entitled "DNA Diagnostics Based on Mass Spectrometry" by H. Köster; Cohen *et al.* (1996) Adv Chromatogr 36:127-162; and Griffin *et al.* (1993) Appl Biochem Biotechnol 38:147-159). It will be evident to one skilled in the art that, for certain embodiments, the occurrence of only one, two or three of the nucleic acid bases need be determined in the sequencing reaction. For instance, A-track sequencing or an equivalent, *e.g.*, where only one nucleotide is detected, can be carried out. Other sequencing methods are known (see, *e.g.*, in U.S. Patent No. 5,580,732 entitled "Method of DNA sequencing employing a mixed DNA-polymer chain probe" and U.S. Patent No. 5,571,676 entitled "Method for mismatch-directed *in vitro* DNA sequencing").

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Please replace the paragraph on page 69, lines 14-25, with the following paragraph:

An example of possible candidate morbidity susceptibility genes are mutants of the A kinase anchoring protein (AKAP) genes. Protein phosphorylation is an important mechanism for enzyme regulation and signal transduction in eukaryotic cells. cAMP dependent protein kinsae (PKA) mediates a variety of hormonal and neurotransmitter responses by phospyhorylating a wide variety of substrates including enzymes, membrane receptors, ion channels and transcription factors. AKAPs direct the subcellular localization of cAMP-dependent protein kinase by binding to its regulatory subunits and therefore play a role in G-protein mediated receptor-signalling pathways. (Huang et al. Proc. Natl. Acad. Sci., USA 94:11184, 1997). AKAPs have a PKA binding region located in their COOH-terminal portion.

Please replace the paragraph on page 88, lines 1-10, with the following paragraph:

Huang et al. Proc. Natl. Acad. Sci. USA, 272:8057-8064 (1997); Protein preparations containing AKAP10 are incubated with glutathione resin in PBS for 2 hours at 4 degrees Celsius with 0.1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 1mM EDTA, 5mM benzamidine, and 5mM β-mercaptoethanol and washed extensively with the same buffer. 200 micrograms of PKA regulatory subunit RII and/or RI were added to the resin and incubated at 4 degrees Celsius. Proteins associated with the AKAP10 are eluted and analyzed by Laemmli electrophoresis. The proteins were visualized by Coomassie Staining. PKA proteins can be radiolabeled or labeled with a flurophore to allow detection.

Please replace the paragraph on page 88, lines 18-28, with the following paragraph:

The PKA assay is typically carried out in a reaction of the enzyme with a peptide substrate and gamma ^{32}P-ATP followed by separation of the ^{32}P-peptide product from the unreacted gamma ^{32}P-ATP on a phosphocellulose membrane. This method requires at least one basic amino acid residue in the peptide substrate. The peptide substrate can be tagged with a biotin group so that the biotinylated ^{32}P-peptide product consistently binds to a streptavidin membrane in a manner independent of the peptide sequence as described in Goueli et al Analytical Biochemistry 225, 10-17, (1995). The separation of the ^{32}P-peptide product from the free gamma ^{32}P-ATP using affinity binding and ultrafiltration separation to analyze a mixture sample as described in U.S. Patent No. 5,869,275.

Please replace the paragraph beginning on page 91, line 23, through page 92, line 21, with the following paragraph:

Ribozymes may be prepared by chemical synthesis or produced by recombinant vectors according to methods established for the synthesis of RNA molecules. See, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), incorporated herein by reference. The ribozyme sequence may be synthesized, for example, using RNA polymerases such as T7 or SP6. The ribozymes may be prepared from a corresponding DNA sequence (DNA which on transcription yields a ribozyme) operably linked to an RNA polymerase promoter such as the promoter for T7 RNA polymerase or SP6 RNA polymerase. A DNA sequence corresponding to a ribozyme may be ligated in to a DNA vector, such as a plasmid, bacteriophage or other virus. Where the transfer vector contains an RNA polymerase promoter operably linked to DNA corresponding to a ribozyme, the ribozyme may be conveniently produced upon incubation with an RNA polymerase. Ribozymes may therefore be produced in vitro by incubation of

RNA polymerase with an RNA polymerase promoter operably linked to DNA corresponding to a ribozyme, in the presence of ribonucleotides. In vivo, prokaryotic or eukaryotic cells (including mammalian cells) may be transfected with an appropriate vector containing genetic material corresponding to a ribozyme, operably linked to an RNA polymerase promoter such that the ribozyme is transcribed in the host cell. Ribozymes may be directly transcribed in vivo from a transfer vector, or alternatively, may be transcribed as part of a larger RNA molecule. For example, DNA corresponding to ribozyme sequence may be ligated into the 3' end of a carrier gene, for example, after a translation stop signal. Larger RNA molecules may help to stabilize the ribozyme molecules against nuclease digestion within the cells. On translation the carrier gene may give rise to a protein, whose presence can be directly assayed if desired, for example, by enzymatic reaction when the carrier gene encodes an enzyme.

Please replace the paragraph on page 95, lines 16-29, with the following paragraph:

Blood was obtained from a donor by venous puncture and preserved with 1mM EDTA pH 8.0. Ten milliliters of whole blood from each donor was centrifuged at 2000x g. One milliliter of the buffy coat was added to 9 milliliters of 155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM Na_2EDTA, incubated 10 minutes at room temperature and centrifuged for 10 minutes at 2000x g. The supernatant was removed, and the white cell pellet was washed in 155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM Na_2EDTA and resuspended in 4.5 milliliters of 50mM Tris, 5mM EDTA, and 1% SDS. Proteins were precipitated from the cell lysate by 6M ammonium acetate pH 7.3 and separated from the nucleic acid by centrifugation at 3000x g. The nucleic acid was recovered from the supernatant by the addition of an equal volume of 100% isopropanol and centrifugation at 2000x g. The dried nucleic acid pellet was hydrated in IOmM Tris pH 7.6 and 1mM Na_2EDTA and stored at 4°C.

Please replace the paragraph on page 96, lines 1-30, with the following paragraph:

AKAP10-1 is an allele of the AKAP10 gene with a single nucleotide polymorphism at nucleotide number 156277 (based on the sequence of a genomic clone of the AKAP10 gene, GenBank Accession No. AC005730). The single nucleotide polymorphism is a T to C transversion located in the 3'non-translated region of the gene encoding AKAP10. PCR primers were synthesized by OPERON (Alameda, CA) using phosphoramidite chemistry. Amplification of the AKAP10 target sequence was carried out in single 50μl PCR reaction with 25ng of human genomic DNA obtained from samples as described in Example 1. Each reaction containing IX PCR buffer (Qiagen, Valencia, CA), 200μM dNTPs, 1U Hotstar Taq polymerase (Qiagen, Valencia, CA), 4mM $MgCl_2$, and 25 pmol of the forward primer containing the universal primer sequence and the target specific sequence 5'-TCTCAATCATGTGCATTGAGG-3' (SEQ ID NO: 5) 2 pmol of the reverse primer 5'-AGCGGATAACAATTTCACACAGGGATCACACAGCCATCAGCAG-3' (SEQ ID NO: 6) and 10pmol of a biotinylated universal primer complementary to the 5' end of the PCR amplicon 5'-AGCGGATAACAATTTCACACAGG-3' (SEQ ID NO: 7). Alternatively, the biotinylated universal primer could be 5'-GGCGCACGCCTCCACG-3' (SEQ ID NO: 16). After an initial round of amplification of the target with the specific forward and reverse primer, the 5' biotinylated universal primer was hybridized and acted as a reverse primer thereby introducing a 3' biotin capture moiety into the molecule. The amplification protocol resulted in a 5'-biotinylated double stranded DNA amplicon, which dramatically reduces the cost of high throughput genotyping by eliminating the need to 5' biotin label each forward primer used in a genotyping. Thermal cycling was performed in 0.2mL tubes or 96 well plate using an MJ Research Thermal Cycler (Waltham, MA) (calculated temperature) with the following cycling parameters: 94°C for 5 min; 45 cycles: 94°C for 20 sec, 56°C for 30 sec, 72°C for 60 sec; 72°C for 3 min.

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Please replace the paragraphs beginning on page 97, line 1, through page 98, line 2, with the following paragraphs:

The 50μl PCR reaction was added to 25μl of streptavidin coated magnetic bead (Dynal) prewashed three times and resuspended in 1M NH_4Cl, 0.06M NH_4OH. The PCR amplicons were allowed to bind to the beads for 15 minutes at room temperature. The beads were then collected with a magnet, and the supernatant containing unbound DNA was removed. The unbound strand was released from the double stranded amplicons by incubation in 100mM NaOH and washing of the beads three times with 10mM Tris pH 8.0.

Genotyping

Genotyping was carried out using the MassEXTEND™ assay and MALDI-TOF. The SNP identified at position 156277 of AKAP10 in the GenBank sequence is represented as a T to C transversion. The MassEXTEND™ assay detected the sequence of the complementary strand at the polymorphic position, thus the primer extension product incorporated either a T or a C. The DNA coated magnetic beads were resuspended in 26 mM Tris-HCl pH 9.5, 6.5 mM $MgCl_2$ and 50 mM each of dTTPs and 50 mM each of ddCTP, ddATP, ddGTP, 2.5U of a thermostable DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) and 20 pmol of a template specific oligonucleotide primer 5'-CTGGCGCCCACGTGGTCAA-3' (SEQ ID NO: 8) (Operon, Alameda, CA). Primer extension occurs with three cycles of oligonucleotide primer hybridization and extension. The extension products were analyzed after denaturation from the template with 50 mM NH_4Cl and transfer of 150 nl each sample to a silicon chip preloaded with 150 nl of H3PA (3-hydroxy picolinic acid) (Sigma Aldrich, St. Louis, MO) matrix material. The sample material was allowed to crystallize and analyzed by MALDI-TOF (Bruker Daltonics, Billerica, MA, PerSeptive, Foster City, CA). The mass of the primer used in the MassEXTEND™ reaction was 5500.6 daltons. The allelic variant results in the addition of ddC to the primer to produce an extension product having a mass of 5773.8 daltons. The predominant allele is extended by the addition of T and

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ddG to the primer to produce an extension product having a mass of 6101 daltons.

Please replace the paragraphs beginning on page 99, line 9, through page 100, line 9, with the following paragraphs:

Genomic DNA was isolated from blood (see Example 1) of seventeen (17) individuals with a genotype CC at the AKAP10-1 gene locus and a single heterozygous individual (CT) (as described in Example 2). A target sequence in the AKAP10-1 gene which encodes the C-terminal PKA binding domain was amplified using the polymerase chain reaction. PCR primers were synthesized by OPERON (Alameda, CA) using phosphoramidite chemistry. Amplification of the AKAP10-1 target sequence was carried out in individual 50μl PCR reaction with 25ng of human genomic DNA templates. Each reaction containing I X PCR buffer (Qiagen, Valencia, CA), 200μM dNTPs, IU Hotstar Taq polymerase (Qiagen, Valencia, CA), 4 mM $MgCl_2$, and 25 pmol of the forward primer containing the universal primer sequence and the target specific sequence 5'-TCC CAA AGT GCT GGA ATT AC-3' (SEQ ID NO: 9), 2pmol of the reverse primer 5'-GTC CAA TAT ATG CAA ACA GTT G-3'(SEQ ID NO:10). Thermal cycling was performed in 0.2 mL tubes or 96 well plate using an MJ Research Thermal Cycler (MJ Research, Waltham, MA) (calculated temperature) with the following cycling parameters: 94°C for 5 min; 45 cycles; 94°C for 20 sec, 56°C for 30 sec, 72°C for 60 sec; 72°C for 3 min. After amplification the amplicons were purified by chromatography (Mo Bio Laboratories (Solana Beach, CA).

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The sequence of the 18 amplicons, representing the target region, was determined using a standard Sanger cycle sequencing method with 25 nmol of the PCR amplicon, 3.2 μM DNA sequencing primer 5'-CCC ACA GCA GTT AAT CCT TC-3' (SEQ ID NO:11) and chain terminating dRhodamine labeled 2', 3' dideoxynucleotides (PE Biosystems, Foster City, CA) using the following cycling parameters: 96°C for 15 sec, 25 cycles: 55°C for 15 sec, 60°C for 4 min.

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The sequencing products were precipitated by 0.3M NaOAc and ethanol, the precipitate was centrifuged and dried. The pellets were resuspended in deionized formamide and separated on a 5% polyacrylamide gel. The sequence was determined using the "Sequencher" software (Gene Codes, Ann Arbor, MI).

Please replace the paragraphs beginning on page 101, line 7, through page 102, line 2, with the following paragraphs:

PCR primers were synthesized by OPERON (Alameda, CA) using phosphoramidite chemistry. Amplification of the AKAP10 target sequence was carried out in single 50μl PCR reaction with 100ng- 1ug of pooled human genomic DNAs in a 50μl PCR reaction. Individual DNA concentrations within the pooled samples were present in equal concentration with the final concentration ranging from 1-25ng. Each reaction contained 1X PCR buffer (Qiagen, Valencia, CA), 200μM dNTPs, 1U Hotstar Taq polymerase (Qiagen, Valencia, CA), 4 mM $MgCl_2$, and 25 pmol of the forward primer containing the universal primer sequence and the target specific sequence

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5'-AGCGGATAACAATTTCACACAGGGAGCTAGCTTGGAAGATTGC-3' (SEQ ID NO:12), 2pmol of the reverse primer 5'-GTCCAATATATGCAAACAGTTG-3' (SEQ ID NO: 10) and 10 pmol of a biotinylated universal primer complementary to the 5' end of the PCR amplicon BIO:5'-AGCGGATAACAATTTCACACAGG-3' (SEQ ID NO: 7). After an initial round of amplification with the target with the specific forward and reverse primer, the 5' biotinylated universal primer can then hybridize and act as a forward primer thereby introducing a 5' biotin capture moiety into the molecule. The amplification protocol resulted in a 5'-biotinylated double stranded DNA amplicon and dramatically reduces the cost of high throughput genotyping by eliminating the need to 5' biotin label every forward primer used in a genotyping.

Themal cycling was performed in 0.2 mL tubes or 96 well plate using an MJ Research Thermal Cycler (Waltham, MA) (calculated temperature) with the

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following cycling parameters: 94°C for 5 min; 45 cycles: 94°C for 20 sec, 56°C for 30 sec; 72°C for 60 sec; 72°C for 3 min.

Please replace the paragraph beginning on page 102, lines 23, through page 103, line 10, with the following paragraph:

The MassEXTEND™ assay detected the sequence of the sense strand and resulted in the incorporation of either T or C into the extension product. The DNA coated magnetic beads were suspended in 26mM Tris-HCl pH 9.5; 6.5 mM $MgCl_2$ and 50mM each of dTTPs and 50mM each of ddCTP, ddATP, ddGTP, 2.5U of a thermostable DNA polymerase (Amersham Pharmacia Biotech, Piscataway NJ) and 20 pmol of a template specific oligonucleotide primer 5'-ACTGAGCCTGCTGCATAA-3' (SEQ ID NO:15) (Operon) (Alameda, CA).

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Primer extension occurs with three cycles of oligonucleotide primer hybridization and extension. The extension products were analyzed after denaturation from the template with 50 mM NH_4Cl and transfer of 150 nl each sample to a silicon chip preloaded with 150 nl of H3PA (3-hydroxy picolinic acid) (Sigma Aldrich, St. Louis, MO) matrix material. The sample material was allowed to crystallize and was analyzed by MALDI-TOF (Bruker Daltonics, Billerica, MA, PerSeptive, Foster City, CA). The primer had a mass of 5483.6 daltons. The allelic variant resulted in the addition of a ddC to the primer to produce an extension product having a mass of 5756.8 daltons. The predominant allele resulted in the addition a T and ddG to the primer giving an extension product with a mass of 6101 daltons.

Please replace the paragraphs beginning on page 104, line 27, through page 105, line 19, with the following paragraphs:

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The identity of the nucleotide present at the polymorphic site of AKAP 10-6 is determined by using the MassEXTEND™ assay and MALDI-TOF (see, U.S. Patent No. 6,043,031). The MassEXTEND™ assay detected the sequence of the sense strand and resulted in the incorporation of either G or C into the